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We have assessed the ability of*Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B to transform mammary epithelial cells. Epitope-tagged Wnt proteins were expressed in cells and the proteins were analyzed by immunoblots. Extracellular heparin-bound forms of Wnt-1, Wnt-3A, and Wnt-5A proteins were detected in culture supernatants. The transforming potential of Wnt proteins was tested using retroviral vectors to express genes in C57MG mammary epithelial cells. Paracrine transforming capability of *Wnt* genes was tested by co-cultivating mammary epithelial cells with *Wnt*-expressing Rat fibroblasts. Immunoblot analysis confirms the expression of Wnt proteins in all cell lines. Direct and paracrine transforming assays indicates that Wnt-1, Wnt-2, Wnt-3 and Wnt-3A proteins transform mammary epithelial cells; Wnt-7A and Wnt-7B proteins partially transform; and Wnt-4, Wnt-5A, Wnt-5B, and Wnt-6 proteins does not affect mammary epithelial cells. Wnt gene family members thus differ in their potential to morphologically transform mammary epithelial cells, suggesting several distinct receptors or quantitative differences in the signals different Wnt proteins provide.

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WNT PROTEINS IN MAMMARY EPITHELIAL TRANSFORMATION
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INTRODUCTION

I. NATURE OF THE PROBLEM

There is strong evidence that Wnt proteins function as peptide growth factors that regulate the mammary gland growth cycle. Some of these proteins have already been shown to contribute to experimental mammary gland tumorigenesis in the mouse. Several groups are currently assessing whether *Wnt* genes play a role in the pathology of human mammary tumors, as might be predicted. Despite this evidence, work focused on the role of *Wnt* genes in breast cancer is in its infancy. The proposed studies are directly aimed at testing the hypothesis that *Wnt* genes encode regulators of normal and neoplastic mammary gland development.

The Wnt-1 protein is recognized as a mediator of cell-cell signaling events that can contribute to mammary tumorigenesis in the mouse. Despite accumulating evidence that Wnt-1 proteins act as growth factors, in the past it has been extremely difficult to purify Wnt-1 proteins in a soluble, cell-free form. For this reason, very little is known about Wnt specific cell surface receptors, which are proposed to be responsible for receiving signals from extracellular Wnt proteins. There is a pressing need to produce soluble, active Wnt ligands in order to understand the nature and regulation of Wnt-mediated growth control. In this proposal we will evaluate the hypothesis that *Wnt* genes encode a family of proteins that act as secreted growth factors that affect mammary epithelial cell physiology by interacting with cell surface receptors. It is expected that several of the Wnt proteins will demonstrably affect the growth properties of mammary epithelial cells, that these proteins act as secreted factors, and that they carry out their functions by stimulating specific cell-surface receptors on mammary epithelial cells.

II. BACKGROUND

The development of the mammary gland is a poorly understood process that consists of cycles of growth, morphogenesis, differentiation, and involution under the control of a variety of hormones and growth factors. On the basis of their ability to affect mammary gland growth and on their expression patterns, several peptide growth factors have been implicated as effectors of mammary gland development (reviewed in (1)). In many cases, deregulation of growth factor-stimulated signaling pathways contributes to the pathobiology of breast cancer (2, 3). The *Wnt* gene family encodes secretory proteins involved in cell growth and cell fate determination during murine embryogenesis, organogenesis, and oncogenesis. We are interested in the role of Wnt proteins in mammary gland development and oncogenesis.

Wnt family genes

The first *Wnt* genes to be cloned were identified based on their oncogenic effects in the mouse mammary gland. The *Wnt*-1 gene (originally *int*-1(4)) was initially identified as a frequent target for insertional activation by mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary gland tumors(5, 6). Inappropriate expression of the *Wnt*-1 gene has been shown to contribute to mammary gland tumorigenesis(7, 8). One other *Wnt* gene, *Wnt*-3, was also originally identified as a transcriptionally activated oncogene in MMTV-induced mammary tumors(9). Most of the identified *Wnt* genes were isolated by

searching for genes homologous to *Wnt*-1 using hybridization techniques(10, 11). and the polymerase chain reaction (PCR)(12). Each of the sequenced open reading frames encodes what appear to be cysteine-rich, secretory glycoproteins ranging from 350-380 amino acids. A comparison of the predicted amino acid sequences among murine *Wnt* gene family members reveal over 100 conserved residues fairly evenly distributed across the entire sequence and striking conservation of roughly 23 cysteines in nearly parallel positions. Different Wnt proteins are generally 40-60% identical at the amino acid level.

The normal functions of *Wnt* genes have been analyzed in several organisms; most extensively in those tractable to genetic or biochemical analysis of early development. Such studies have shown that Wnt proteins are involved in diverse developmental phenomena. The *Wnt*-1 orthologue in *Drosophila* is the segment polarity gene *wingless* (13, 14). A combination of genetic and biochemical analyses, suggests that the wg protein functions as a local-acting, secreted factor that triggers a cascade of molecular events leading to the specification of segment polarity in the *Drosophila* embryo(15, 16, 17, 18). The wg protein has also been shown to have organizer activities that lead to specification of spatial patterns in adult structures, such as leg or wing(19, 20) and it is also involved in regulating neuroblast specification in the *Drosophila* central nervous system(21). In the frog, *Xenopus laevis,* several different *Wnt* genes have been shown to contribute to the experimental induction of dorsal mesoderm tissue and subsequent establishment of the body axis (22, 23, 24, 25). Current models of early embryonic patterning events in the frog propose the involvement of one or several Wnt proteins as determinants of dorsal axial position(25, 26). The murine *Wnt* genes cloned to date are expressed in spatially restricted patterns during gastrulation, neurulation, or early organogenesis. Of the *Wnt* genes analyzed, seven of the family members show restricted expression patterns in the developing brain, and several other family members are expressed in the neural tube and neural plate(27). On the basis of the analysis of *Wnt*-1 gene deficiencies, the normal function of the murine *Wnt*-1 gene is in proper development of the cerebellum and midbrain (28, 29, 30, 31). These observations have led to the proposal that murine Wnt proteins act either as mitogens or act to specify cell fate in the developing nervous system. Wnt proteins are also implicated in the process of limb development(27). Several *Wnt* genes are expressed in the developing limb and ectopic expression of *Wnt*-1 in developing limbs of transgenic mice results in abnormalities in growth and skeletal patterning of the limb(32).

Wnt proteins and their mechanism of action

The predicted primary protein of the *Wnt*-1 gene displays many of the characteristics of secreted growth factors: a hydrophobic signal peptide, a recognition site for signal peptidase, prospective sites of N-linked glycosylation, many cysteine residues, and lack of any identifiable membrane anchor domain(33). Due to the lack of cell lines expressing the endogenous *Wnt*-1 gene, most of the work on the biochemical properties of Wnt proteins has been carried out with cells programmed to express exogenous *Wnt* cDNAs. In these ectopic settings, Wnt-1 proteins behave as secretory glycoproteins, undergoing entry into the endoplasmic reticulum (ER), leader cleavage, and asparagine(N)-linked glycosylation at several sites(34, 35). Despite entry into the ER, Wnt-1 proteins are very poorly secreted. Most of the Wnt-1 protein remains associated with internal membranous components of cells. Intracellular Wnt-1 is predominantly bound to BiP; a chaperonin-like protein found in the ER(36). A small portion of the most highly glycosylated forms of Wnt-1 proteins is secreted(37, 38). The appearance of extracellular Wnt-1 proteins is significantly enhanced by addition of heparin sulfate(37) or suramin(39) to the media. Such experiments suggest that

Wnt-1 proteins that have moved through the secretory pathway into the extracellular environment are not freely diffusible, but instead are tightly associated with either the cell surface(39) or the extracellular matrix(37). Although Wnt-1 proteins are not readily detected freely soluble in the media of cells expressing *Wnt*-1 cDNA, evidence has accumulated that Wnt proteins can act in a paracrine fashion. First, entry into the secretory pathway is necessary for Wnt-1 biological activity(22, 40). Second, cell transformation assays have been developed that depend on paracrine effects of Wnt-1(40, 41). These paracrine assays involve co-cultivation of cells that do not exhibit responses to *Wnt*-1 expression (fibroblast non-responsive cells) and mammary epithelial cell lines. When *Wnt*-1 responsive cells (C57MG) are mixed with or surround Wnt-1 donor cells, they undergo morphological changes. Finally, analysis of wg protein function, the *Drosophila* homologue of *Wnt*-1, suggests that it acts in a paracrine fashion since the *wg* mutant phenotype is cell non autonomous(42); that is, mutant cells can be rescued by surrounding wild-type cells. These observations have led to the model that Wnt-1 proteins are local-acting factors that function to signal to cells that are adjacent or near the site of Wnt production but do not affect cells at sites distant from the site of production. In fact, Wnt-1 proteins tethered to the cell surface by addition of a transmembrane tail still exhibit autocrine and paracrine transforming activities(43, 44). Recently, it has been reported that Wnt-1 protein activity can be detected in the media of mammary epithelial cells programmed to express a *Wnt*-1 cDNA (A.M.C. Brown, personal communication; J. Kitajewski, unpublished observations) suggesting that Wnt proteins can also act as diffusible secreted growth factors.

The Wnt-1 protein is now recognized as a mediator of cell-cell signaling events. Little is known regarding Wnt cell surface receptors or the nature of the signaling events triggered by receptor activation. Some clues have come from analysis of the wg signal transduction pathway in Drosophila embryos. *wg* expression is known to influence the expression of at least two homeobox-containing genes, *Distal-less* and *engrailed*(16). *wg* also affects the subcellular localization or levels of protein products encoded by another segmentation polarity gene, *armadillo*(*arm*). Armadillo is similar to the vertebrate proteins plakoglobin and b-catenin(45, 46), which are found associated with cadherins in desmosomes and adherens junctions. Wnts may therefore regulate the association of plakoglobin or b-catenin to the cadherin family of molecules or alternately cadherins and catenins may participate in transmitting Wnt-induced signals(47).

***Wnt* genes and mammary tumorigenesis**

Abnormal expression of the *Wnt*-1 gene products contributes to the development of mammary tumors(16). Transgenic mice expressing the *Wnt*-1 gene in the mammary gland develop mammary tumors and these tumors have high levels of *Wnt*-1 mRNA(7). Expression of the *Wnt*-1 gene in two established mammary epithelial cell lines, C57MG cells(48) or RAC311C cells(49)leads to morphological transformation from flat cuboidal cells to highly refractile, elongated cells that continue to grow after confluence. In one of these cell lines, RAC cells, *Wnt*-1 expression leads to increased tumorigenicity of the cells. In contrast, primary embryo cells and several established rodent fibroblast cell lines do not respond to *Wnt*-1 expression. To date, *Wnt*-1 mediated transformation appears to be restricted to mammary epithelial cells. There is also evidence that *Wnt* genes can contribute to mouse mammary tumorigenesis by gene amplification and resulting overexpression(9, 50). These studies have established that the *Wnt* genes are potent oncogenes in mouse mammary tumorigenesis.

Several lines of evidence suggest that the proteins encoded by the *Wnt* gene family may affect mammary gland development. Mice bearing a *Wnt*-1 transgene that is expressed in the mammary gland exhibit extensive hormone-independent hyperplasia of mammary epithelium(7). In these mice, the glands of both virgin females and male animals resemble those of pregnant animals, and ovarectomy and adrenalectomy have no obvious effect on the morphology of these mammary hyperplasias(51). Both *Wnt*-1 and *Wnt*-3 expression can affect mammary gland growth; however, neither gene is expressed in the normal mammary gland. Since the mammary gland responds to both *Wnt*-1 and *Wnt*-3, it has been proposed that they act through Wnt specific cell surface receptors found on mammary epithelial cells and that these receptors normally respond to proteins encoded by other *Wnt* gene family members that are expressed in the mammary gland. In fact, as shown in table 1, several *Wnt* genes are found to be expressed during post-natal development of the mammary gland(52, 53). Of the cloned and published *Wnt* genes, *Wnt*-2, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, and *Wnt*-7A are expressed in the mammary gland during periods of mammary gland growth and differentiation (in virgin and pregnant glands). In lactating glands, when the gland is no longer growing, none of the identified *Wnt* genes are expressed. These findings suggest that regulated expression of *Wnt* gene products may play a role in the normal expansion or differentiation of the mammary epithelium before lactation. The oncogenic effects of the *Wnt*-1 and *Wnt*-3 genes may thus interfere with the normal *Wnt*-mediated regulation of mammary gland growth.

Human *Wnt* Genes 2, 3, 4, and 7B have been found to be expressed in human breast cell lines and disease states of human breast tissue when compared to normal breast tissue. These results provide a strong rationale for studies on the action of Wnt proteins as a means of understanding the normal and neoplastic development of the mammary gland.

Table 1. Summary of temporal expression of *Wnt* genes in the mouse mammary gland*

		Pregnancy			Lactation	
	Virgin	Early	Mid	Late	Early	Late
Wnt-1	-	-	-	-	-	-
Wnt-2	+**	-	-	-	-	-
Wnt-3	-	-	-	-	-	-
Wnt-3A	-	-	-	-	-	-
Wnt-4	+	+	+	+	+	-
Wnt-5A	-	+	+	+	-	-
Wnt-5B	-	+	+	+	-	-
Wnt-6	-	+	+	+	+	-
Wnt-7A	-	-	-	-	-	-
Wnt-7B	+	+	+	-	-	-

*From reference (17) ** From reference (53)

III. PURPOSE

The *overall objective* of the work proposed here is to determine how Wnt proteins modulate the growth of mammary epithelial cells, with the *long term goal* of understanding the role of *Wnt* genes in mammary tumorigenesis.

IV. METHODS OF APPROACH

Our *general strategy* is to carry out a study of the proteins encoded by ten different *Wnt* genes (*Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, *Wnt*-7B) that will address the following *specific aims:*

1. *Examine the biochemical and secretory properties of Wnt proteins.* The coding potential for an antigenic epitope has been added to full length cDNAs encoding *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B. We will prepare stable cell lines expressing epitope-tagged Wnt proteins in order to determine how the biochemical properties of the proteins encoded by newly described *Wnt* genes compare to those described for Wnt-1 proteins. We will evaluate if these proteins enter the secretory pathway, how efficiently are they secreted, and once outside the cell are these proteins freely soluble, bound tightly to the extracellular matrix, or bound to the cell surface? Our goal is to identify Wnt proteins that can be purified for use as ligands.

2. *Determine the transforming potential of Wnt genes.* Using retroviral vectors to express the proteins encoded by these cDNAs in cultured cell lines, we have determined whether: (a) expression of these genes in cultured mammary epithelial cells leads to transformation, and (b) these proteins transmit signals in a paracrine fashion.

BODY

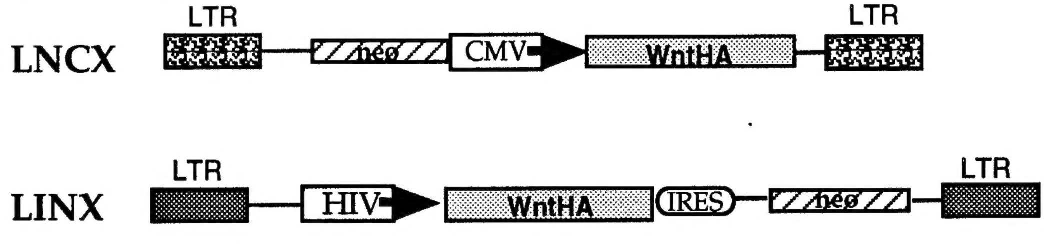
I. EXPERIMENTAL METHODS/RESULTS

Biochemical and secretory properties of Wnt proteins.

Epitope-tagging Wnt proteins. A key feature of the work proposed is to develop methods for the immunological detection and purification of proteins encoded by the *Wnt* gene family. Rather than generate antibodies against each of the Wnt proteins we have tagged Wnt proteins with an antigenic epitope for which well characterized, specific antibodies have already been generated; a process referred to as epitope tagging. Among the advantages of using epitope-tagged Wnt proteins in the experiments to be described is the ability to detect ectopically expressed Wnt proteins without the complications of detecting endogenously expressed Wnt proteins. The epitope we have chosen to add to Wnt proteins has the amino acid sequence YPYDVPDYA derived from the influenza HemAgglutinin (HA) protein and is recognized by monoclonal antibody 12CA5 (54)(the 12CA5 antibody will be referred to as the anti-HA antibody). In order to create vectors for the expression of epitope-tagged Wnt proteins, we have used site-directed mutagenesis to generate cDNAs encoding Wnt proteins fused in frame at their C-terminus to the amino acid sequence MA**YPYDVPDYA**SLGPGP (the bold letters represent residues recognized by the anti-HA antibody). *Wnt* cDNAs were first subcloned into phagemid vectors (pBluescript from Promega) that have the sequences encoding the HA epitope situated downstream. Single strands were generated from the phagemid and used in a site-directed mutagenesis protocol with an oligonucleotide designed to loop-out the sequences between the last codon in the *Wnt* sequence and the first codon of the HA peptide to create a cDNA encoding the fusion protein. **Figure 1**, following page (pg. 8) displays an alignment of the amino acid sequences of Wnt proteins included in the analysis and the amino acid sequence of the added HA-epitope.

These cDNAs were then be subcloned into two different murine leukemia virus (MLV) based vectors, denoted LNCX vectors(55) and LINX . The LNCX vectors utilize the cytomegalovirus immediate early promoter/enhancer to drive expression of the gene of interest and the retroviral LTR to drive expression of the *neo* gene, which confers resistance to the drug G418. LNCX vectors were used to generate Rat fibroblast cell lines expressing different Wnt genes. LINX vectors were developed in our laboratory and utilize the HIV LTR as an internal promoter driving a bicistronic mRNA with a Wnt cDNA, followed by a poliovirus internal ribosome entry site (IRES), followed by a *neo gene.* We have also generated control retroviral vectors. As a positive control, we have used a vector expressing *Wnt*-1 cDNA with no epitope-tag; this vector has been shown to be transforming. As negative controls, we have used the parental vector that does not contain a *Wnt* gene. **Figure 2**, shown below, displays the structure of the two retroviral vectors.

Figure 2. Schematic diagram of retroviral vectors, LNCX and LINX



```
WNT-1   --------------M GLWALLPSWVSTTLL LALTALPAALAANSS GRWWGIVNIASSTNL L-TDSKSLQLVLEPS LQLLSRKQRRLIRQN
WNT-2   --------------- -------------MN VPLGGIWLWLPL--L LTWLTPEVSSSWWYM RATGG-SSRVMCDNV PGLVSR-QRQLCHRH
WNT-3   --------------- -----------MEPH LLGLLLGLLLSGTRV LAGYPIWW-SLALGQ QYTSLASQPLLCGSI PGLVPK-QLRFCRNY
WNT-3A  --------------- --------------M APLGYLLVLCSLKQA LGSYPIWW-SLAVGP QYSSLSTQPILCASI PGLVPK-QLRFCRNY
WNT-4   --------------- -----------MSPR SCLRSLRLLVFA-VF SAAASNWL-YLAKLS SVGSI-SEEETCEKL KGLIQR-QVQMCKRN
WNT-5A  MKKPIGILSPGVALG TAGGAMSSKFFLMAL ATFFSFAQVVIE--A NSWWSLGMNNPVQMS EVYII-GAQPLCSQL AGLSQG-QKKLCHLY
WNT-5B  ------MLVPGHWDG -LRPAMPSLLLVVVA ALLSSWAQLLTD--A NSWWSLAL-NPVQRP EMFII-GAQPVCSQL PGLSPG-QRKLCQLY
WNT-6   --------------- --------------- -MLPPVPSRLGL-LL LLLCPAHVDGLWWAV GSPLVMDPTSICRKA RRLAGR-QAELCQAE
WNT-7A  --------------- --------------- -MTRKARRCLGH--L FLSLGIVYLRIGGFS SVVAL-GASIICNKI PGLAPR-QRAICQSR
WNT-7B  --------------- --------------- -MHRNFRKWIFY--V FLCFGVLYVKLGALS SVVAL-GANIICNKI PGLAPR-QRAICQSR

WNT-1   PGILHSVSGGLQSAV RECKWQFRNRRWNCP T-APGPHLFGKIVNR GCRETAFIFAITSAG VTHSVARSCSEGSIE SCTCDY---------
WNT-2   PDVMRAIGLGVAEWT AECQHQFRQHRWNCN TLDRDHSLFGRVLLR SSRESAFVYAISSAG VVFAITRACSQGELK SCSCDPK--------
WNT-3   IEIMPSVAEGVKLGI QECQHQFRGRRWNCT TIDDSLAIFGPVLDK ATRESAFVHAIASAG VAFAVTRSCAEGTST ICGCDS---------
WNT-3A  VEIMPSVAEGVKAGI QECQHQFRGRRWNCT TVSNSLAIFGPVLDK ATRESAFVHAIASAG VAFAVTRSCAEGSAA ICGCSS---------
WNT-4   LEVMDSVRRGAQLAI EECQYQFRNRRWNCS TL-DSLPVFGKVVTQ GTREAAFVYAISSAG VAFAVTRACSSGELE KCGCDR---------
WNT-5A  QDHMQYIGEGAKTGM KECQYQFRHRRWNCS TVD-NTSVFGRVMQI GSRETAFTYAVSAAG VVNAMSRACREGELS TCGCSR---------
WNT-5B  QEHMSYIGEGAKTGI RECQHQFRGRRWNCS TVD-NTSVFGRVMQI GSRETAFTYAVSAAG VVNAISRACREGELS TCGCSRA--------
WNT-6   PEVVAELARGARLGV RECQFQFRFRRWNCS SH---SKAFGRVLQQ DIRETAFVFAITAAG ASHAVTQACSMGELL QCGCQAPRGRAPPRP
WNT-7A  PDAIIVIGEGSQMGL DECQFQFRNGRWNCS ALG-ERTVFGKELKV GSREAAFTYAIIAAG VAHAITAACTQGNLS DCGCDK---------
WNT-7B  PDAIIVIREGAQMGI DECQHQFRFGRWNCS ALG-EKTVFGQELRV GSREAAFTYAITAAG VAHAVTAACSQGNLS NCGCDR---------

WNT-1   --RRRGP-------- --GGPDWHWGGCSDN IDFGRLFGREFVDSG E-----------KGR DLRFLMNLHNNEAGR TTVFSEMRQECKCHG
WNT-2   ----------KKGSA KDSKGTFDWGGCSDN IDYGIKFARAFVDAK ER-----KG--KDAR A---LMNLHNNRAGR KAVKRFLKQECKCHG
WNT-3   ------------HHK GPPGEGWKWGGCSED ADFGVLVSREFADAR EN-----RP---DAR S---AMNKHNNEAGR TTILDHMHLKCKCHG
WNT-3A  ------------RLQ GSPGEGWKWGGCSED IEFGGMVSREFADAR EN-----RP---DAR S---AMNRHNNEAGR QAIASHMHLKCKCHG
WNT-4   ------------TVH GVSPQGFQWSGCSDN IAYGVAFSQSFVDVR ER-----SK---GAS SSRALMNLHNNEAGR KAILTHMRVECKCHG
WNT-5A  ------------ARP KDLPRDWLWGGCGDN IDYGHPFAKEFVDAR ERERIHAKGSYESAR I---LMNLHNNEAGR RTVYNLADVACKCHG
WNT-5B  ------------ARP KDLPRDWLWGGCGDN VEYGYRFAKEFVDAR EREKNFAKGSEEQGR A---LMNLQNNEAGR RAVYKMADVACKCHG
WNT-6   SGLLGTPGPPGPTGS PDASAAWEWGGCGDD VDFGDEKSRLFMDAQ HK-----RG---RGD IR-ALVQLHNNEAGR LAVRSHTRTECKCHG
WNT-7A  ----------EKQGQ YHWDEGWKWGGCSAD IRYGIGFAKVFVDAR EI-----KQ---NAR T---LMNLHNNEAGR KILEENMKLECKCHG
WNT-7B  ----------EKQGY YNQAEGWKWGGCSAD VRYGIDFSRRFVDAR EI-----KK---NAR R---LMNLHNNEAGR KVLEDRMKLECKCHG

WNT-1   MSGSCTVRTCWMRLP TLRAVGDVLRDRFDG ASRVLYGN--RGSNR ASRAELLRLEPEDPA HKPPSPHDLVYFEKS PNFCTYSGRLGTAGT
WNT-2   VSGSCTLRTCWLAMA DFRKTGEYLWRKYNG AIQVVMNQDGTG--- --------FTVANKR FKKPTKNDLVYFENS PDYCIRDREAGSLGT
WNT-3   LSGSCEVKTCWWAQP DFRAIGDFLKDKYDS ASEMVVEKHRESRGW VE-----TLRAKYAL FKPPTERDLVYYENS PNFCEPNPETGSFGT
WNT-3A  LSGSCEVKTCWWSQP DFRTIGDFLKDKYDS ASEMVVEKHRESRGW VE-----TLRPRYTY FKVPTERDLVYYEAS PNFCEPNPETGSFGT
WNT-4   VSGSCEVKTCWRAVP PFRQVGHALKEKFDG ATEVEPRRVGSSRAL V---------PRNAQ FKPHTDEDLVYLEPS PDFCEQDIRSGVLGT
WNT-5A  VSGSCSLKTCWLQLA DFRKVGDALKEKYDS AAAMRLNSRGK---- --------LVQVNSR FNSPTTQDLVYVDPS PDYCLRNETTGSLGT
WNT-5B  VSGSCSLKTCWLQLA EFRKVGDRLKEKYDS AAAMRITRQGK---- --------LELANSR FNQPTPEDLVYVDPS PDYCLRNETTGSLGT
WNT-6   LSGSCALSTCWQKLP PFREVGARLLERFHG ASRVMGTNDGK---- -------ALLPAVRT LKPPGRADLLYAADS PDFCAPNRRTGSPGT
WNT-7A  VSGSCTTKTCWTTLP QFRELGYVLKDKYNE AVHVEPVRASRNKRP TF-----LKIKKPLS YRKPMDTDLVYIELS PNYCEEDPVTGSVGT
WNT-7B  VSGSCTTKTCWTTLP KFREVGHLLKEKYNA AVQVEVVRASRLRQP TF-----LRIKQLRS YQKPMETDLVYIEKS PNYCEEDAATGSVGT
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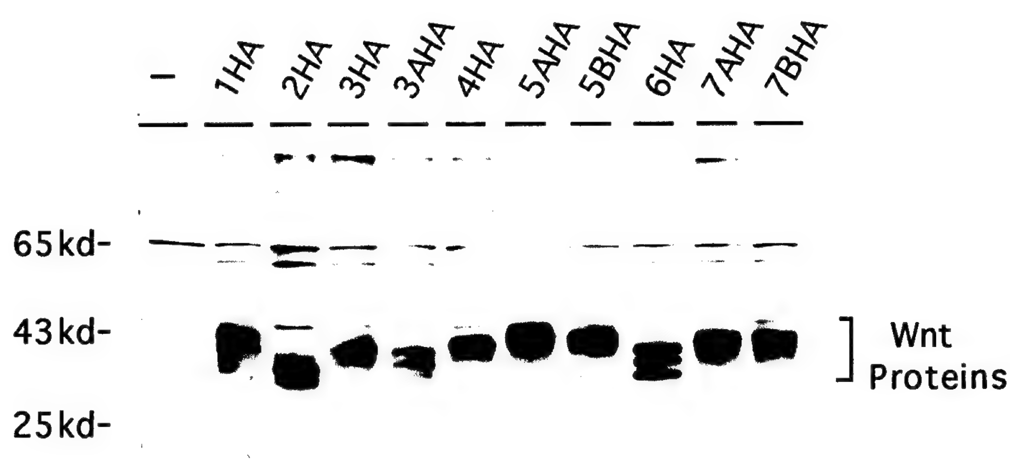
WNT-1	AGRACNSSSPALDGC	ELLCCGRGHRTRTQR	VTERCNCTFHWCCHV	SCRNCTHTRVLHECL	**SMAYPYDVPDYASLGPGL**	387
WNT-2	AGRVCNLTSRGMDSC	EVMCCGRGYDTSHVT	RMTKCECKFHWCCAV	RCQDCLEALDVHTCK	APKS ADWATPT**SMAYPYDVPDYASLGPGL**	377
WNT-3	RDRTCNVTSHGIDGC	DLLCCGRGHNARTEK	RKEKCHCVFHWCCYV	SCQECIRIYDVHTCK	**SMAYPYDVPDYASLGPGL**	372
WNT-3A	RDRTCNVSSHGIDGC	DLLCCGRGHNTRTER	RREKCHCVFHWCCYV	SCQECTRVYDVHTCK	**SMAYPYDVPDYASLGPGL**	369
WNT-4	RGRTCNKTSKAIDGC	ELLCCGRGFHTAQVE	LAERCGCRFHWCCFV	KCRQCQRLVEMHTCR	**SMAYPYDVPDYASLGPGL**	368
WNT-5A	QGRLCNKTSEGMDGC	ELMCCGRGYDQFKTV	QTERCHCKFHWCCYV	KCKKCTEIVDQFVCK	**SMAYPYDVPDYASLGPGL**	396
WNT-5B	QGRLCNKTSEGMDGC	ELMCCGRGYDRFKSV	QVERCHCRFHWCCFV	RCKKCTEVVDQYVCK	**SMAYPYDVPDYASLGPGL**	389
WNT-6	RGRACNSSAPDLSGC	DLLCCGRGHRQESVQ	LEENCLCRFHWCCVV	QCHRCRVRKELSLCL	**SMAYPYDVPDYASLGPGL**	381
WNT-7A	QGRACNKTAPQASGC	DLMCCGRGYNTHQYA	RVWQCNCKFHWCCYV	KCNTCSERTEMYTCK	**SMAYPYDVPDYASLGPGL**	366
WNT-7B	QGRLCNRTSPGADGC	DTMCCGRGYNTHQYT	KVWQCNCKFHWCCFV	KCNTCSERTEVFTCK	**SMAYPYDVPDYASLGPGL**	366

Figure 1. Alignment of amino acid sequences of Wnt proteins with HA epitope at C-terminus (Engineered epitope = bold, HA epitope= bold/underlined).

Analysis of the biochemical and secretory properties of Wnt proteins was carried out in cell lines programmed to express these genes that were generated by infection with retroviral expression vectors. High-titer, helper free retroviral stocks were generated using the BOSC23 ecotropic virus packaging cell line(56). This cell line produces high titer retroviral stocks after transient transfection with retrovirus encoding plasmids (>10^5 colony forming units/ml) allowing rapid generation cell lines. The plasmids bearing Wnt-HA retroviral expression vectors were transfected into BOSC-23 cells, media was collected, and used to infect either C57MG mammary epithelial or Rat-1 fibroblast cell lines. These cell lines will be used to both analyze Wnt proteins (*Specific aim* 1) and to assess autocrine and paracrine transforming potential (*Specific aim* 2, see below).

Characterization of Wnt proteins. We have identified Wnt proteins in cell lines that are programmed to ectopically express the HA-tagged Wnt proteins. Protein expression was first evaluated in transiently transfected 293T cells. Cultured cells were transfected with 10 µg of vector DNA and two days later protein expression was evaluated by immunoblot analysis to demonstrate that these cells are producing Wnt proteins. Cells were lysed in detergent (1% Triton-X100, in 10mm tris, 100mmNaCl), insoluble material was removed, and proteins in the lysate were fractionated on SDS-gels, transferred to nitrocellulose, and then subjected to immunoblot analysis using the anti-HA antibody. Proteins were be visualized on the immunoblots using Enhanced Chemiluminescence (ECL, Amersham). Extracts from control vector-infected cells were be used as a negative control for the immunoblot analysis.

Figure 3. Immunoblot of HA tagged Wnt proteins produced in transfected 293T cells.

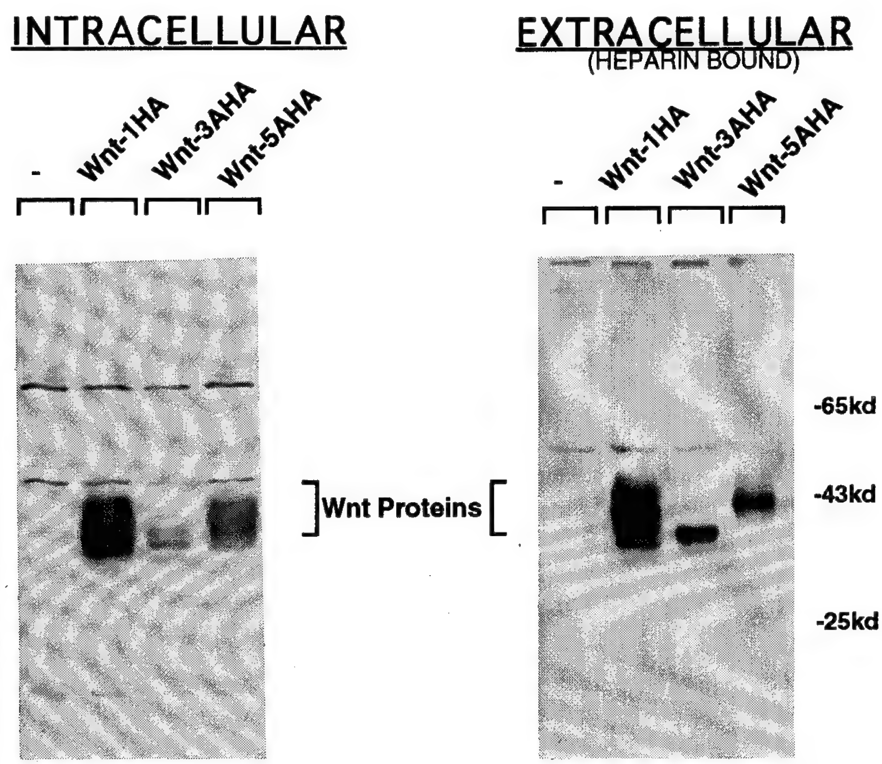


The size of the Wnt proteins and the number of species detected is consistent with that predicted from the primary amino acid sequence of the ten Wnt proteins and the potential glycosylation sites identified in the sequence. In addition, roughly comparable levels of expression was seen for the ten Wnt proteins.

Analysis of secreted Wnt proteins. Extracellular Wnt-1 proteins are generally detected only after addition of compounds that can compete growth factors off extracellular

material. A number of growth factors and other secreted proteins are thought to associate with the extracellular matrix because of their affinity for heparin and/or heparin sulfate glycosaminoglycan. Extracellular Wnt-1 proteins can be detected as heparin-bound proteins (37). We have determined whether other Wnt proteins can be detected as heparin-bound species. Transiently transfected 293T cells expressing HA-tagged Wnt proteins were incubated 1 day post-transfection for 24 hours either in the absence or presence of soluble heparin-sulfate (100μg/ml). The media from the cells was spun at 10k X g for 10 minutes to remove intact cells, and the resulting media was be centrifuged at 100k X g for 2 hours to pellet the heparin-sulfate. The heparin-sulfate pellet was resuspended in Laemli sample buffer and the proteins bound to heparin were analyzed by immunoblot analysis. We have detected extracellular heparin-bound forms of HA-tagged Wnt-1, Wnt-3A, and Wnt-5A proteins produced by transiently transfected cells (Figure 4).

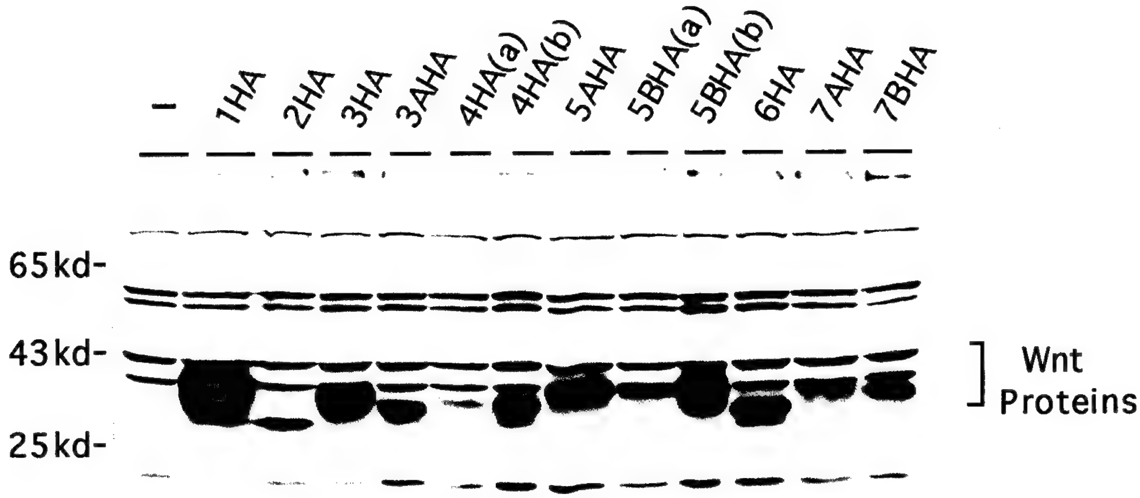
Figure 4. Immunoblot analysis of Wnt-1, Wnt-3A, and Wnt-5A proteins produced in 293T cells. Extracellular, heparin-bound forms and intracellular forms.



The secretory potential of Wnt-1, Wnt-3A, and Wnt-5A proteins appears roughly equivalent. In order to detect these proteins, heparin addition was required indicating that each is bound to extracellular material.

Generation of Rat-1 fibroblasts expressing Wnt proteins. We and others have developed assays that specifically test for biological effects dependent on the secretion of Wnt-1 proteins. In these assays, paracrine activity is supplied by cell lines that are programmed to express Wnt proteins but which do not themselves detectably respond to these proteins (e.g., mouse 3T3 fibroblasts, Rat-1 fibroblasts, or HeLa cells). When mammary epithelial cells are placed in proximity to Wnt-1 donor fibroblast cell lines, the mammary epithelial cells become morphologically transformed. In order to test the paracrine transforming potential of *Wnt* gene family members we developed a panel of Rat-B1A fibroblasts that express the ten epitope-tagged Wnt proteins. For expression in Rat-B1A cells we have found that the CMV promoter efficiently drives expression of foreign genes; therefore we used a panel of LNCX based vectors bearing Wnt-HA cDNAs. The plasmids vectors were transfected into BOSC-23 cells, media was collected two days post-transfection, and used to infect Rat-B1A fibroblast cell lines. Selection (400μg/ml G418) was added two days after infection and colonies were pooled approximately 1 week after selection. Pooled colonies were used as a source of extracts to evaluate protein expression by immunoblot analysis, as described above. **Figure 5**, displays an immunoblot analysis of cell lines programmed to express Wnt-1, Wnt-2, Wnt-3, Wnt-3A, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A, Wnt-7B.

Figure 5



All of these cells were expressing the epitope-tagged Wnt proteins. In the case of Wnt-4 and Wnt-5B, we repeated the generation of the cell line in a bid to get cells expressing higher amounts of Wnt protein; Wnt-4(b) and Wnt-5B(b) were cell lines developed that had higher levels of intracellular Wnt proteins.

The transforming potential of *Wnt* genes.

Wnt-1 and *Wnt*-3 behave as mammary oncogenes when expressed in the mouse mammary gland. We know little about the biological activities of the proteins encoded by the newly identified *Wnt* genes, some of which are normally expressed in the developing mammary gland (see Table 1). The goal of*specific aim* 2 is to use mammary epithelial transformation assays as a measure of the biological activity of Wnt proteins. We have compared the transforming activities of a subset of the murine *Wnt* gene family that includes *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B. These include genes expressed in the developing mammary gland (*Wnt*-2, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, and *Wnt*-7B) as well as genes not normally expressed in the mammary gland (*Wnt*-1, *Wnt*-3, *Wnt*-3A, and *Wnt*-7A).

Autocrine transformation by *Wnt* gene family members. To determine whether a *Wnt* gene has the potential to transform mammary epithelial cells, we have used retroviral vectors to generate cell lines ectopically expressing Wnt proteins (described in*specific aim* 1) and assessed the morphology and growth properties of these cells. Autocrine transforming potential was tested using the murine C57MG cell line(57). We have used the C57MG cell line extensively to study *Wnt*-1 mediated transformation. The HIV LTR appears to function as a better promoter than the CMV promoter in the C57MG cell line (unpublished observations); therefore, we used a panel of LINX vectors with the ten epitope-tagged Wnt cDNAs to develop C57MG cell lines.

C57MG cells were infected with retroviral vectors, as described above, to generate cell lines programmed to express HA-tagged Wnt proteins (see*specific aim* 1). The transformed phenotype in these cells was evaluated by visibly assessing the morphology of confluent cultures, the results of the direct transformation assay are tabulated in Table II, and are discussed below.

Paracrine transformation by Wnt gene family members. Wnt-1 proteins can affect mammary epithelial cells in a paracrine fashion(40, 41). We have determined if the proteins encoded by *Wnt* gene family members also act as paracrine effectors of mammary epithelial cell growth by generating Rat-B1A fibroblast cell lines that ectopically express HA-tagged Wnt proteins, see above and Figure 5. Paracrine assays were done by co-culturing*Wnt*-expressing Rat-1 cell lines with C57MG cells. The negative control in these paracrine transformation assays will be co-cultivation of uninfected Rat-1 cells with C57MG cells; this resulted in a flat monolayer composed of both cell types. The positive control was co-cultivation of *Wnt*-1 expressing Rat-1 cells with C57MG cells, which results in morphological transformation of the mammary epithelial cells. Since the transformed cells continue to grow post-confluence the result of this assay is a culture that appears completely transformed. The results of this assay are tabulated in Table II. Our results indicate that HA-tagged Wnt-1, Wnt-2, Wnt-3, and Wnt 3A transform mammary epithelial cells. HA-tagged Wnt-7A and Wnt-7B gene expression leads to weak transformation of mammary epithelial cells. Finally, Wnt-4, Wnt-5A, Wnt-5B, and Wnt-6 do not detectably transform mammary epithelial cells. These experiments also confirm that, in the case of Wnt-1, addition of the HA-epitope does not measurably affect the biological activity of these proteins. Paracrine assays were carried out for all ten Wnt genes using a set of Rat-B1A fibroblast cell lines as donors of Wnt activity. The results of the paracrine assays coincide with results obtained by direct expression in mammary epithelial cells. Our results indicate that there are two classes of Wnt proteins as evaluated by our assays in mammary epithelial cells; transforming Wnt genes and non-transforming Wnt genes. The Wnt proteins that are transforming all have the ability to

transform cells in a paracrine fashion demonstrating that the *Wnt* gene family encodes paracrine-acting growth factors.

Table II. Oncogenicity, Mammary gland expression, transforming potential of Wnt gene family members.

	mammary gland	oncogenic	transforming direct	transforming paracrine
Wnt-1	-	+	+	+
Wnt-2	+ virgin, ducts		+	+
Wnt-3	-	+	+	+
Wnt-3A	-		+	+
Wnt-4	+ virgin, pregnant		-	-
Wnt-5A	+ pregnant		-	-
Wnt-5B	+ pregnant		-	-
Wnt-6	+ pregnant		-	-
Wnt-7A	-		+	+
Wnt-7B	+ virgin, pregnant		-/+	-/+

II. Goals of the Research

The data presented in this annual report represents results of experiments outlined in specific aim 1 and specific aim 2 of the research proposal. We feel we have completed approximately three-fourths of the work toward these goals and are currently evaluating the secretory potential of the Wnt proteins and determining if activity can be detected from conditioned media of cells expressing Wnt proteins. We have clearly segregated Wnt family members into two functional classes based upon their ability to transform cultured mammary epithelial cells.

CONCLUSIONS

In conclusion, we have segregated Wnt proteins into functional classes based upon their ability to transform mammary epithelial cells. This segregation may represent classes of Wnt proteins that interact with distinct Wnt-cell surface receptors. This may represent the first type of evidence that their may be distinct Wnt-cell surface receptors. Alternatively, one class may be involved in mitogenic stimulus and are thus are transforming, whereas the other class may be involved in differentiation of the mammary epithelium. Two interesting aspects of the segregation come out of this analysis. First, it appears that those Wnt genes either not normally expressed in the mammary gland (Wnt-1, Wnt-3, Wnt-3A, and Wnt-7B) or expressed at very low levels in the mammary gland (Wnt-2) are the most transforming. Whereas, those Wnt genes that are well expressed in the mammary gland (Wnt-4, Wnt-5A, Wnt-5B, Wnt-6) do not exhibit transformation activity. Second, when one compares the activity of the transforming genes to those reported to be overexpressed in mammary tumors(58) only one Wnt gene that is not transforming is overexpressed in this study. Wnt-2, Wnt-3, and Wnt-7B were all found to be transforming in our hands and have been found to be overexpressed in several mammary tumors; however, Wnt-4 never displayed transforming activity in our experiments but was found to be overexpressed in mammary tumors. Future work will be focused on assessing the activity of these proteins as soluble factors, identifying domains required for transforming activity, and searching for Wnt receptors in mammary epithelial cells.

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